

# THE EFFECTS OF ELECTROLYTES AND SUGARS ON THE ERYTHROCYTES OF THE TURTLE, *CHELYDRA* *SERPENTINA*<sup>1</sup>

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The erythrocytes of the snapping turtle, *Chelydra serpentina*, exhibit a particular sensitivity to the lack of calcium and will hemolyze in electrolyte solutions which do not contain this ion, though not in pure glucose (Lyman, 1945). The absence of hemolysis reported for glucose is presumed to be due to impermeability to the glucose molecule because of its size. If this is true, sugars of smaller molecular volume might prove hemolytic. Two factors other than size may also have an influence. From Gibbs-Donnan equilibrium relationships, changes in membrane structure may be expected when a non-electrolyte replaces an electrolyte medium, all sugars having an equivalent action in this respect. In addition, sugars might affect the cell membrane by direct chemical action, and such effects may be characteristic for the individual sugars. With regard to the hemolytic action of calcium-free electrolyte solutions on *Chelydra* erythrocytes, it is not known to what extent electrolytes differ in their action, nor in what manner an alteration of the hydrogen ion concentration of any given solution may influence the hemolytic process. These various factors have been considered in the present study.

The results show that hemolysis occurring in calcium-free electrolyte solutions is influenced by the ionic composition of the medium. Moreover, by suitable adjustment of the hydrogen ion concentration, the integrity of the cell membrane may be maintained for short periods even in the absence of calcium. In an examination of the effects of isosmotic solutions of various sugars, striking differences were found, some being hemolytic and others not. Agglutination and hemolytic reactions observed in certain sugars indicate that these compounds are not inert, but produce a definite alteration of the cell membrane.

## MATERIAL AND METHODS

Blood (0.5 — 1.0 ml.) of *C. serpentina* drawn without anticoagulant was washed twice in 40 ml. of frog Ringer and suspended in Ringer. In determining the rate of hemolysis, washed cells were centrifuged briefly; the supernate was very carefully removed, and 5.0 ml. of experimental solution added to give a cell concentration of approximately 1:100. The optical density was measured within the first minute and at intervals with a Fisher electrophotometer (Wilbur and Collier, 1943). A Beckman spectrophotometer, made available through the kindness of Dr. W. J. Dann, was used for determinations of the optical density of hemoglobin solutions and for the hemoglobin spectrum of turtle blood.

<sup>1</sup> Aided by a grant from the Duke University Research Council.

Hematocrit tests showed frog Ringer to be approximately isotonic with slight variations between individuals. Experimental solutions were made isosmotic with 0.125 molal NaCl. Double distilled water was used throughout. Galactose, xylose and arabinose were Pfanstiehl brand and were free of calcium. NaCl was Merck Reagent For Biological Work. Other chemicals were reagent grade and were not further purified.

We wish to thank Dr. M. H. Jacobs and Dr. H. B. Collier for their helpful suggestions and Mr. N. G. Anderson and Mr. R. L. Rigsbee for photographic work.

## RESULTS

### *Electrolytes*

The observation of Lyman (1945) that erythrocytes of *C. serpentina* will hemolyze in isotonic Ca-free salt solutions was readily confirmed. Moreover the course of hemolysis in isosmotic NaCl, for example, could be arrested by the addition of a small amount of isosmotic  $\text{CaCl}_2$  solution to the hemolyzing suspension. However, in preliminary experiments it became apparent that the rate of hemolysis varies with the cation and anion employed and also with the hydrogen ion concentration.

The effect of different cations was examined by following the course of hemolysis in buffered and unbuffered isosmotic solutions of NaCl, KCl,  $\text{MgCl}_2$  and  $\text{CaCl}_2$ . Hemolysis was always most rapid in KCl, followed by NaCl, slower in  $\text{MgCl}_2$  (Figs. 1 and 2), and completely absent in  $\text{CaCl}_2$ .

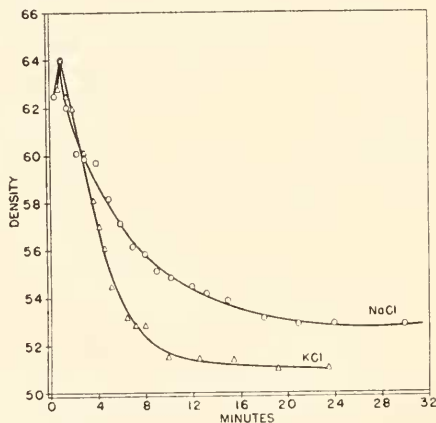


FIGURE 1. Hemolysis of *Chelydra* erythrocytes in 0.125 molal NaCl and 0.126 molal KCl. 29° C. pH 7.41. Solutions buffered with corresponding isosmotic phosphate in the proportion 9:1. The initial rise in the curves indicates preliminary shrinkage probably due to slight hypertonicity. The final values for optical density represent complete hemolysis for both solutions, the difference apparently resulting from difference in opacity of the ghosts.

The rate of hemolysis in any given electrolyte solution was always greater in an alkaline than in an acid solution. This could be shown by adjusting the pH with HCl or the hydroxide of the cation being studied, or by use of phosphate buffers. Figure 3 illustrates the effect in the case of KCl buffered with isosmotic phosphate.

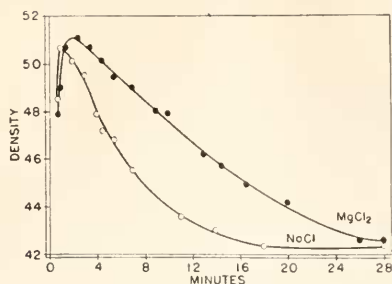


FIGURE 2. Hemolysis of *Chelydra* erythrocytes in 0.125 molal NaCl and 0.088 molal  $\text{MgCl}_2$ . 28° C. pH 7.41. Solutions buffered with 0.1 molal sodium phosphate 9:1.

NaCl behaved similarly. The rate showed little change within the range pH 7.8 to pH 6.7 but was definitely decreased at pH 5.9 to pH 6.1. The inhibitory effect of acidity was demonstrated in another manner (Fig. 4). Cells were placed in NaCl buffered to pH 7.0 with a trace of phosphate. After hemolysis was well under way acidified NaCl was added. Hemolysis was quickly arrested. On the addition of alkaline NaCl hemolysis was resumed at the original rate, indicating reversibility of the inhibition. If cells remain in the acid NaCl longer than about 10 minutes, hemolysis will be resumed at a very slow rate. When the pH is restored to the alkaline range after 50 minutes the former rapid rate is regained.

The effects of the cyanide and citrate of sodium and potassium were compared with the corresponding chloride at pH 7.25 or 7.4. Isosmotic mixtures of chloride and cyanide containing 0.019 molal cyanide exhibited a hemolytic action similar to that of isosmotic chloride. The same results were obtained with cells exposed to

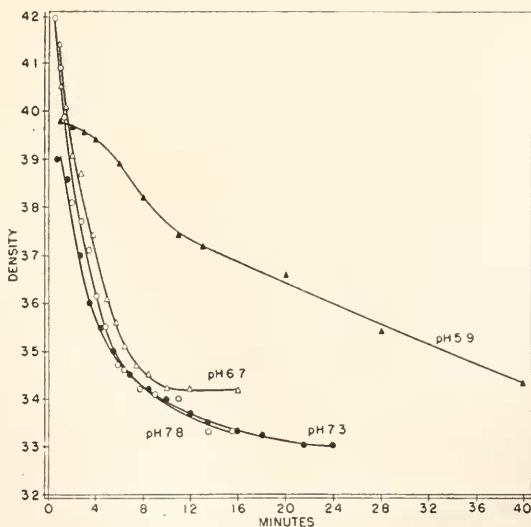


FIGURE 3. Hemolysis of *Chelydra* erythrocytes in 0.126 molal KCl buffered with 0.1 molal potassium phosphate 9:1. 28° C.

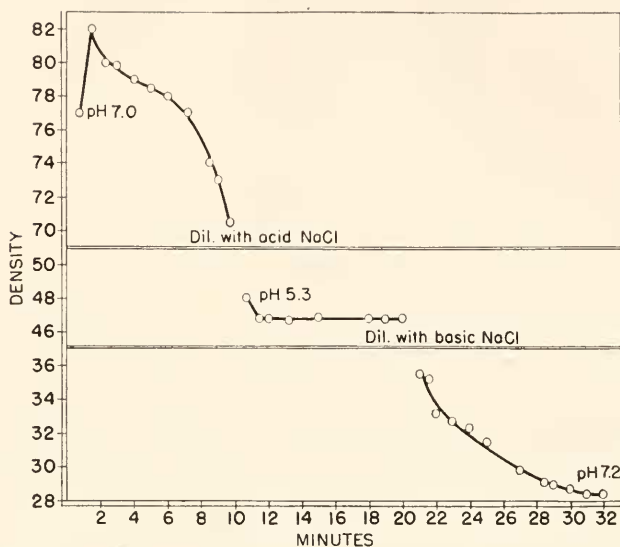


FIGURE 4. Effect of acidity on hemolysis of *Chelydra* erythrocytes in 0.125 molal NaCl. Ten minutes after the erythrocytes were mixed with 4.0 ml. NaCl solution (pH 7.0), and during the course of rapid hemolysis, 3.0 ml. of acid NaCl was added bringing the pH to 5.3. Hemolysis was arrested within one minute. Addition of 1.9 ml. of alkaline NaCl at 20 minutes raised the pH and hemolysis continued immediately. Dilution gave a marked change in optical density as indicated. The initial rise in the curve indicates shrinkage probably due to hypertonicity. 21° C.

the same concentration of cyanide in Ringer for 30 minutes before adding the hemolytic solution. The absence of any marked effect is not surprising in view of previous work with other electrolytes (Davson and Danielli, 1938; Hunter, 1947). Isosmotic sodium citrate gave slower hemolysis than NaCl, which may be the result of impermeability to the citrate ion as compared with chloride (Jacobs, 1940).

### *Sugars*

When *Chelydra* erythrocytes are suspended in unbuffered isosmotic dextrose solution, agglutination occurs followed by hemolysis. The amount of hemolysis can be determined by removing the cells by centrifugation and measuring the concentration of hemoglobin in the solution. If the suspension is pipetted up and down, the masses of cells tend to break up and hemolysis is further increased. In dextrose buffered at pH 7.5 with a small amount of sodium phosphate, agglutination was no longer apparent; and, as might be expected from the effect of pH on hemolysis in salt solutions, hemolysis was more pronounced.<sup>2</sup> A comparison of the hemolytic action of various sugars, including one disaccharide, two hexoses, and two pentoses (Table I), showed that the pentoses differed markedly from the other sugars, hemolysis being absent in arabinose and very slight in xylose. Dextrose gave complete hemolysis. On centrifugation of such hemolyzing suspensions, a

<sup>2</sup> The buffer may well have an effect as an electrolyte.

jelly-like mass containing nuclei, many distorted and a few normal erythrocytes, was found. This contrasts with hemolysis in electrolytes in which normal ghosts were present, with about 50 per cent of the ghosts spherizing in isosmotic  $\text{MgCl}_2$ .

Observations on agglutination indicate that differences exist between sugars in this respect as well as in their hemolytic action. Cells (0.07–0.08 ml.) suspended in Ringer were centrifuged briefly; the supernate was carefully removed and 8.5 ml. of isosmotic sugar solution was added. After stirring to give uniform cell distribution, the suspension was left undisturbed.<sup>3</sup> Results were similar for three individuals (Table I). While strict comparisons between certain sugars cannot be made because of differences in pH, it is apparent that all sugars cause agglutination, but all are not equivalent. Moreover, the effects of agglutination do not parallel hemolytic actions. So, for example, sucrose is hemolytic and weakly agglutinating; arabinose, on the other hand, has a greater agglutinating action without producing hemolysis.

TABLE I

*Hemolysis and agglutination in isosmotic sugar solutions*

Cells were removed from solutions after 3 hours and the optical density of the supernate was measured at 576 millimicrons.\* pH 7.5–25° C. Blood from three turtles.

	Hemolysis		Agglutination	
	Sample 1	Sample 2		pH
dextrose	0.630	0.510	++++	5.4
sucrose	0.344	0.378	+	5.7
d-galactose	0.325	0.163	+	4.5
d-xylose	0.087	0.017	++++	4.4
l-arabinose	0.007	0.005	+++	5.7
Ringer	0.017	0.014	0	5.7

\* After initial mixing the suspensions were left undisturbed to minimize hemolysis and then centrifuged. Centrifugation may increase the hemolysis slightly. (Collier, 1948. Personal communication.) The absorption spectrum was determined for one sample of blood, and maxima were located at 543 and 576 millimicrons. These agree closely with values given for human blood.

The influence of electrolytes on agglutination may be shown by adding one part of isosmotic  $\text{NaCl}$ ,  $\text{KCl}$ ,  $\text{MgCl}_2$  or  $\text{CaCl}_2$  to 14 parts of isosmotic dextrose. Agglutination was inhibited somewhat by the addition of electrolytes in all cases and was less pronounced in the presence of  $\text{NaCl}$  and  $\text{MgCl}_2$  than with the other salts (Fig. 5). But in no instance was agglutination completely prevented. Microscopic observations indicate similar differences (Figs. 6 and 7). Quantitative aspects of this effect, previously studied on other cells (Radsma, 1918), have not been investigated.

<sup>3</sup> Sedimentation rate fails to give a measure of the extent of agglutination in this blood inasmuch as some cells do not agglutinate and therefore sediment relatively slowly, whereas the agglutinated masses in the same suspension fall rapidly or adhere to the wall of the tube. Adherence to the wall is observed in vertical tubes of 1.0 cm. or more in diameter.

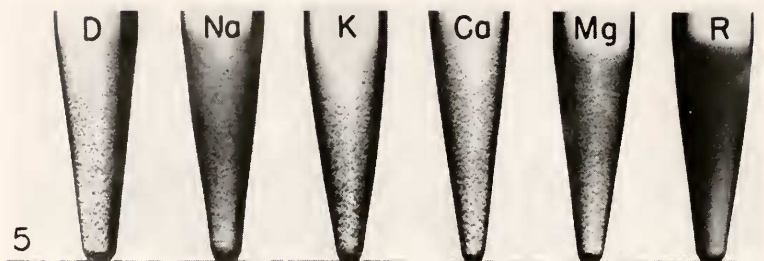


FIGURE 5.



FIGURE 6.



FIGURE 7.

FIGURE 5. Effect of salts on agglutination of washed *Chelydra* erythrocytes in dextrose solution. Isosmotic salt solutions were added to unbuffered dextrose (0.23 molal) in the proportion 1:14. D—dextrose; Na—NaCl + dextrose; K—KCl + dextrose; Ca—CaCl<sub>2</sub> + dextrose; Mg—MgCl<sub>2</sub> + dextrose; R—Ringer (unbuffered). After initial mixing tubes were left undisturbed. 26.5° C.

FIGURE 6. *Chelydra* erythrocytes in 0.23 molal unbuffered dextrose. Washed cells were mixed with dextrose solution and placed immediately on a slide without coverslip. Arrows indicate ghosts.

FIGURE 7. *Chelydra* erythrocytes in 0.23 molal unbuffered dextrose with isosmotic NaCl 14:1. Shrinkage may be noted.

#### DISCUSSION

Consideration of the mode of action of electrolytes and non-electrolytes on *Chelydra* erythrocytes is complicated by the fact that related substances may be strikingly different in their actions. Thus, no single scheme will serve to explain completely the action of sugars, nor can cation effects be interpreted adequately in

terms of valence. The latter is in contrast to human erythrocytes, in which ions of the same valence are alike in preventing loss of salts from cells in sucrose solution (Wilbrandt, 1940).

The effect of calcium in maintaining the normal permeability characteristics of the cell may be considered in relation to (1) the thickness of the ionic double layer and the adhesion of membrane components, and (2) crossbinding of anions within the membrane (Danielli, 1937, 1943). In both respects calcium has a more pronounced action than sodium or potassium. Following the Gibbs-Donnan equilibrium, with a change of medium from Ringer to isosmotic NaCl or KCl as was done in the present experiments, there will be a replacement of calcium in the surface layer. Compactness of the membrane will be decreased, which may in turn lead to an increase in permeability resulting in swelling and hemolysis. Such an increase in volume preceding hemolysis in these solutions may be readily observed under the microscope. Additional assumptions will be necessary to explain such differences as those found between the effects of calcium and magnesium on hemolysis and on sphering of ghost cells.

With sugar solution as the medium, the salt concentration essentially zero, and a constant anion concentration in the cell surface, there is to be expected from the Gibbs-Donnan equilibrium a decrease in membrane concentration of mono- and divalent metal ions, and an increase in surface acidity, which has been thought to be of sufficient magnitude to alter the proteins of the membrane and accordingly cell permeability (Danielli, 1937; Wilbrandt, 1940). However, in this cell an increase in acidity of the medium stabilizes the membrane, as shown by the acid inhibition of hemolysis in both electrolytes and non-electrolytes. At the same time the loss of metal ions would result in increased repulsive forces within the membrane, giving greater distances between molecules and an increase in permeability (Danielli, 1943). Even though it is assumed that the net effect favors an increased permeability, the present results are not completely explained inasmuch as all sugars should behave similarly, whereas some have been shown to be hemolytic while others are not.

Other factors to be considered are a differential permeability to sugars and effects of individual sugars on membrane structure. Any differences in permeability which may exist must involve factors other than molecular volume since this does not correlate with hemolytic action. So, for example, sugars which were least hemolytic (pentoses) have the smallest molecular volume. (See also Ulrich, 1934.) The agglutination of cells indicates an alteration of the cell surface by the sugar, the degree to which this occurs depending upon the particular sugar and ionic composition of the medium. Further, hemolysis in sugar, contrary to the results in electrolyte solutions, was characterized by disintegration of many of the cells, again pointing to a direct action on membrane structure.

The effect of acidity in decreasing hemolysis obtained with electrolyte solutions suggests that molecular rearrangements within the membrane may in part compensate for the lack of calcium. The fact that the erythrocyte is stable in certain Ca-free non-electrolytes indicates that it is not the absence of calcium *per se* which causes hemolysis in electrolytes, but rather the effect of other cations which may replace the calcium of the cell membrane and so increase its permeability. The cell, then, would be sensitive to lack of calcium only because of the ready replacement of its calcium by other cations.

## SUMMARY

1. The comparative hemolytic rates of *Chelydra serpentina* erythrocytes in isosmotic salt solutions as measured photometrically were  $\text{KCl} > \text{NaCl} > \text{MgCl}_2$ , and  $\text{NaCl} > \text{Na}_3\text{ citrate}$ . Hemolysis in cyanide (0.019 molal) was similar to that in chloride. No hemolysis occurred in isosmotic  $\text{CaCl}_2$  and the addition of  $\text{CaCl}_2$  to cells hemolyzing in Ca-free electrolyte solutions arrested hemolysis at once.

2. Hemolysis in sodium and potassium solutions was greatly retarded at about pH 6 and below.

3. The hemolytic potency of isosmotic sugar solutions (pH 7.4) was found to be: dextrose  $>$  sucrose  $>$  d-galactose  $>$  d-xylose with complete hemolysis in dextrose and none in l-arabinose in three hours.

4. Sugar hemolysis was accompanied by abnormal shape changes and disintegration of cells, whereas in Ca-free electrolyte solutions "normal" ghosts were found.

5. Agglutination occurred in unbuffered isosmotic sugar solutions, the extent depending upon the particular sugar. Agglutinating action was not correlated with hemolytic potency.

6. Results of experiments on the hemolytic and agglutinating properties of sugars indicate that certain sugars are not inert but have a definite action on the cell surface.

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